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Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice

(neurotrophins/adenovirus)

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ABSTRACT **Long-term potentiation (LTP) has been shown to be impaired in mice deficient in the brain-derived neurotrophic factor (BDNF) gene, as well as in a number of other knockout animals. Despite its power the gene-targeting approach is always fraught with the danger of looking at the cumulative direct and indirect effects of the absence of a particular gene rather than its immediate function. The re-expression of a specific gene at a selective time point and at a specific site in gene-defective mutants presents a potent procedure to overcome this limitation and to evaluate the causal relationship between the absence of a particular gene and the impairment of a function in gene-defective animals. Here we demonstrate that the re-expression of the BDNF gene in the CA1 region almost completely restores the severely impaired LTP in hippocampal slices of BDNF-deficient mice. The results therefore provide strong evidence for the direct involvement of BDNF in the process of LTP.**

Neurotrophic factors are normally conceived as molecules that influence survival and differentiation of specific populations of neurons during embryonic development through their respective receptors (1–3). Recently, however, evidence has been accumulating that, in addition, neurotrophins can play an important role in postnatal activity-dependent plasticity in the developing mammalian brain (4–9). Neurotrophins can have direct effects on synaptic transmission (10–15) and especially one neurotrophin, brain-derived neurotrophic factor (BDNF), has been shown to be upregulated by physiological stimuli (16), as well as stimuli that lead to hippocampal long-term potentiation (LTP) (17, 18). Moreover, recent evidence shows that hippocampal LTP in the CA1 region of BDNF-mutant mice is severely impaired (19) suggesting that BDNF might be causally involved in the generation of LTP. To ascertain that the deficiency in LTP is not caused by subtle developmental abnormalities undetectable with the methods used in the previous study (19) we re-expressed the BDNF gene in the CA1 region using an adenovirus construct. Here we demonstrate that this procedure "rescues" LTP in hippocampal slices from BDNF-mutant mice.

MATERIALS AND METHODS

Hippocampal transverse slices (400 μm thick) were prepared from wild-type ([+/+]), heterozygous ([+/−]), and homozygous ([−/−]) mice and maintained using standard procedures (medium: 124 mM NaCl/3 mM KCl/1.25 mM KH_2PO_4/2 mM Mg_2SO_4/26 mM $NaHCO_3$/2.5 mM $CaCl_2$/10 mM glucose, temperature 32 ± 0.2°C, submerged recording). Synaptic field potentials were elicited with a frequency of 0.1 Hz. Responses were recorded in the apical dendritic region of the CA1 pyramidal neurons. The slope of the excitatory postsynaptic potential (EPSP) was calculated and used as a measure for synaptic strength. LTP was induced with a tetanus of 3 × 30 pulses (100 Hz, 60 μs duration, 5 s ISI) with the strength of the test stimulus. Routinely, a second pathway was recorded as a control in the same slice. Data were collected with a program written in LABVIEW (National Instruments, Austin, TX).

All tests were done for all three genotypes: Homo- and heterozygous BDNF-deficient mice and their wild-type siblings (SV 129 strain), age: [−/−] mice: P18–P28, [+/−] mice: P18–P52 and [+/+] mice: P18–P52. The measurements were carried out and analyzed in a strictly blind fashion—the injections of the slices with Ad-CMV-LacZ, Ad-CMV-NGF, or Ad-CMV-BDNF [adenoviral vectors coding for β-galactosidase (LacZ), nerve growth factor (NGF), or BDNF] were done by a second experimenter so that the investigator performing the electrophysiological measurements had no way of telling from which type of slice he was recording. This information was only made available to the first experimenter after the experiment was completely analyzed.

Initial tests of the adenoviral vectors were performed in cultures of dissociated hippocampal neurons prepared from wild-type E17 Wistar rats, since under these conditions the amount of virus added to each well could be exactly controlled. One million hippocampal neurons in a minimal volume of 1 ml of defined medium (20) were infected with either 6 × 10^6 plaque-forming units (pfu) of Ad-CMV-NGF or Ad-CMV-BDNF. At the indicated time points after infection total RNA was prepared for Northern blot analysis (21) from controls and infected cultures. A replication-deficient E1-deleted adenovirus Ad-CMV-LacZ was constructed so that it contains an expression cassette consisting of the *Escherichia coli* β-galactosidase gene under control of the cytomegalovirus (CMV) promoter, inserted between nucleotides 452 and 3328 of the genome of mutant dl309 (22). Ad-CMV-BDNF and Ad-CMV-NGF were constructed similarly to Ad-CMV-LacZ, only that the LacZ gene was replaced by either the human prepro-BDNF cDNA (Ad-CMV-BDNF) or the mouse prepro-NGF cDNA (Ad-CMV-NGF). Virus stocks were purified by CsCl density gradient centrifugation (23) and dialyzed against 10 mM Tris, pH 7.6/10% glycerol. Synthesis of bioactive BDNF was demonstrated by infecting hippocampal neurons with Ad-CMV-BDNF. One day after infection the cells were depolarized for 30 min using 50 mM KCl. Supernatants were

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Abbreviations: BDNF, brain-derived neurotrophic factor; EPSP, excitatory postsynaptic potential; LTP, long-term potentiation; CMV, cytomegalovirus; NGF, nerve growth factor; LacZ, β-galactosidase; PPF, paired-pulse facilitation.

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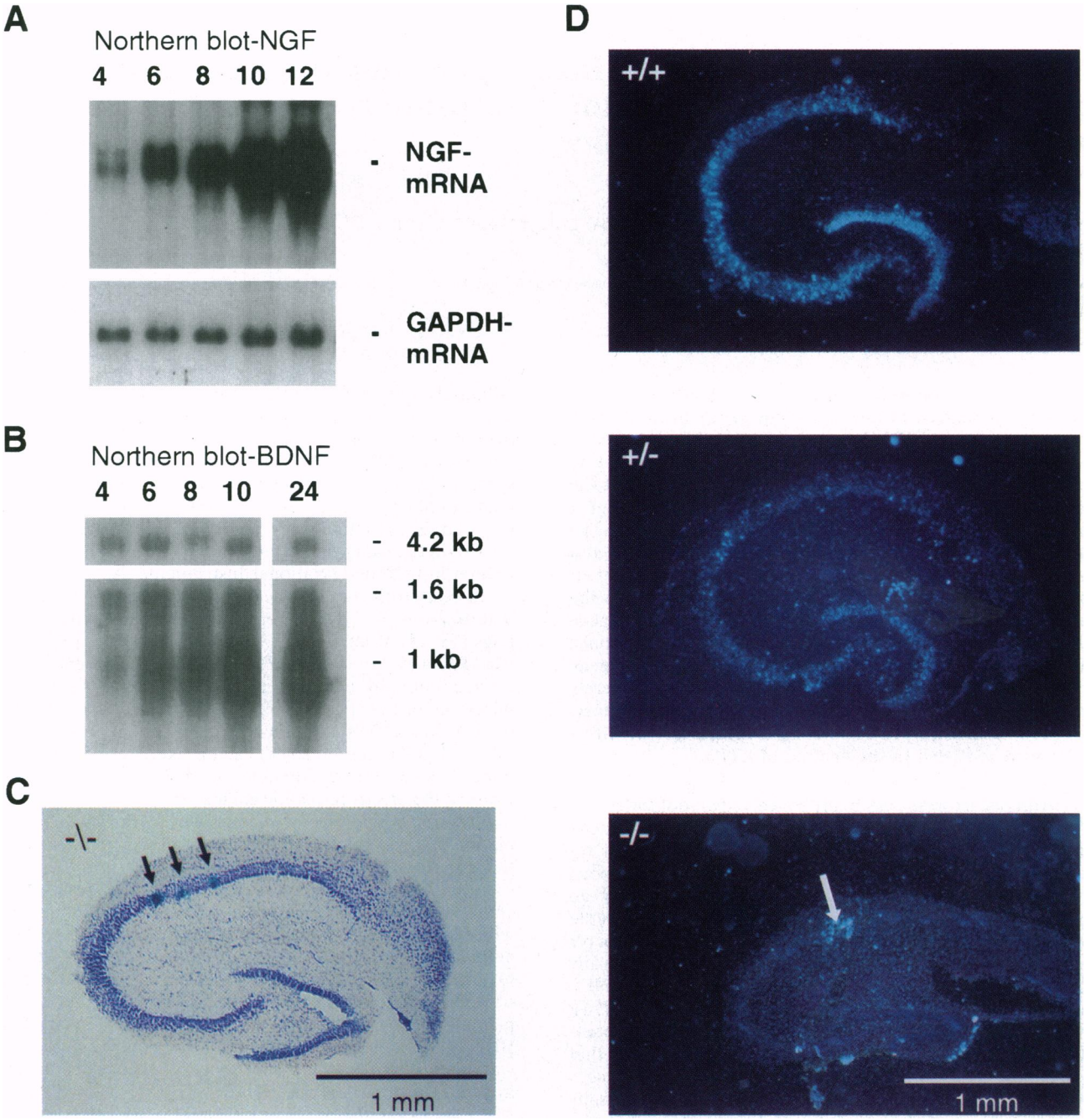

FIG. 1. Infection of slices with recombinant adenoviral vectors. (*A* and *B*) Time course for the expression of NGF and BDNF mRNA (4–12 h and 4–24 h, respectively). This shows that the onset of expression of the transgene after adenoviral infection occurs fast enough to allow rapid gene transfer. (*A*) Initially, NGF mRNA was used because NGF mRNA concentration is extremely low in the hippocampus; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. (*B*) Time course of BDNF mRNA from wild-type animals. The 1.6- and 4.2-kb bands show endogenous BDNF mRNA. The 1-kb band is the Ad-CMV-BDNF mRNA. Between 4 and 6 h after infection the BDNF mRNA transcript from the viral expression cassette is clearly higher than endogenous BDNF mRNA (1.6- and 4.2-kb bands). (*C*) X-gal stain showing the injection site (arrows) of LacZ adenoviral vector 6 h after infection (counterstained with Nissl). This panel shows that the adenoviral infection occurs spatially well restricted. (*D*) *In situ* hybridization for BDNF mRNA in hippocampal slices from [+/+], [+/−], and [−/−] mice 8 h after infection time. One sees a clear dosage effect of the amount of BDNF mRNA from [+/+] through [+/−] to [−/−] animals. In particular, the [−/−] animals show no BDNF mRNA except for the site where the vector had been injected (arrow).

collected and tested by bioassay on chicken nodose neurons. Ad-CMV-NGF was tested by ELISA using antibodies recognizing exclusively native NGF (24).

For the injection procedure slices were placed in a carbogenated interface chamber with a hole in the lid of the chamber. The purified recombinant adenovirus (LacZ, BDNF, or NGF) was then injected into the extracellular space of the CA1 pyramidal cell body layer by pressure injection through a patch pipette (in some experiments alternatively into the CA3 region). The solution contained 2×10^{10} pfu for the Ad-CMV-BDNF (BDNF gene inserted) 1×10^{9} pfu for Ad-CMV-NGF (NGF gene inserted) and $3–5 \times 10^{9}$ pfu for the Ad-CMV-LacZ (β-galactosidase gene inserted). The solution also contained alcian blue to facilitate detection of the infection spot for the electrophysiological recordings. After injection, slices were placed in a carbogenated submerged chamber for up to 14 h at 35°C before the electrophysiological experiment started. After the experiments the slices infected with the LacZ-expressing

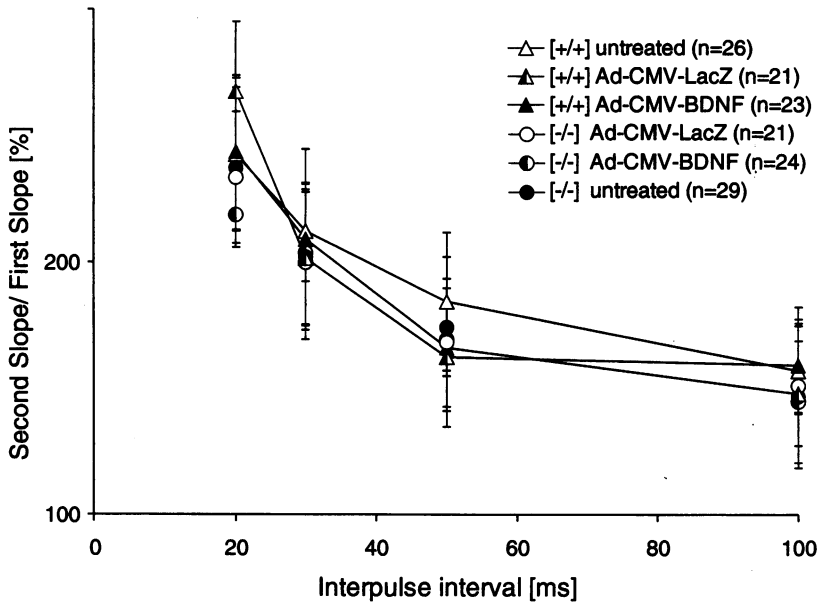

FIG. 2. PPF is not affected in Ad-CMV-BDNF-injected slices. PPF was measured to determine whether this aspect of synaptic transmission was normal in adenovirus-injected slices in comparison to untreated control slices. The percentages denote the ratio of the second EPSP slope to the first EPSP slope. Data are shown for [+/+] and [−/−] slices. PPF was tested with 20-, 30-, 50-, and 100-ms inter-pulse intervals. n = number of slices.

adenovirus were fixed in 4% paraformaldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). For *in situ* hybridization, 15-μm-thick transverse sections were cut on a cryostat and processed as described (25).

RESULTS

The goal of our experiments was to use viral gene transfer for a "rescue experiment" and to re-express the BDNF gene by locally infecting cells in the CA1 region of the hippocampus of wild-type and BDNF-mutant mice, just before testing their ability to undergo LTP. Different viral vectors have been used to transfer genes into a variety of neuronal cells *in vitro* and *in vivo* (26–32), but because of their efficiency, the high titers that can be obtained, and their relatively low cytotoxicity, we chose adenoviral vectors for our experiments.

Previous *in vivo* studies using adenovirus constructs have always focused on achieving long-term expression, with initial time points of expression rarely studied before 24 h after injection. Because in our experiments a significant expression of the delivered gene has to be achieved within the lifetime of the acute slice (which normally does not exceed 20 h) we examined the time course of transgene expression after adenoviral infection. BDNF and NGF mRNA were detectable as early as 4 h after infection with Ad-CMV-BDNF or Ad-CMV-NGF and increased steadily thereafter, demonstrating that the onset of expression was fast enough to allow rescue experiments within the time frame of the viability of acute hippocampal slices (Fig. 1 *A* and *B*). We then micro-injected purified adenoviral stock solutions locally into slices, establishing a local source of BDNF production and release in the CA1 region. Control slices injected with Ad-CMV-LacZ were stained using X-gal (Fig. 1*C*). Furthermore, we performed *in situ* hybridization for BDNF mRNA in [+/+], [+/−], and [−/−] slices (Fig. 1*D*). A clear dosage effect for the amount of BDNF mRNA is visible for the different genotypes. The [−/−] slice shows no BDNF mRNA except for one spot where it was infected with Ad-CMV-BDNF by local injection of the vector. Fig. 1 *C* and *D Bottom* also document that depending on the volumina injected (15–30 nl), locally restricted infection can be achieved within a sphere of 50–200 μm diameter. Taken together, Fig. 1 then shows the feasibility of the adenoviral rescue approach by demonstrating the high efficacy and short delay of gene expression after virus-mediated gene-transfer, which are important prerequisites for combining the rescue approach with electrophysiological measurements.

Table 1. Percentage of successful LTP inductions for [+/+], [+/−] and [−/−]-mice

	Ad-CMV-lacZ		Ad-CMV-NGF		Ad-CMV-BDNF	
	Yes	No	Yes	No	Yes	No
[+/+]	14 (61%)	9	7 (64%)	4	14 (67%)	7
[+/−]	6 (20%)	24	2 (25%)	6	16 (52%)	15
[−/−]	4 (19%)	17	1 (20%)	4	11 (46%)	13

LTP was judged successful if 60 min after the tetanus the slope of the field EPSP was greater than 120% of the baseline value.

Before attempting subtle electrophysiological measurements it is important to establish whether basal synaptic transmission is affected by the infection procedure. We found infected slices to be indistinguishable from untreated control slices for the following three parameters: (*i*) Equivalent responses were evoked with similar stimulus strength. There were no significant differences between different genotypes and different treatments ($P > 0.15$ for all combinations of genotypes and treatments, two-tailed t test, n = 23–94); (*ii*) The area under the field EPSP produced by a single afferent volley was equal in injected and untreated slices ($P > 0.29$ for all combinations of genotypes and treatments, two-tailed t test, n = 23–94); (*iii*) Paired-pulse facilitation (PPF) was normal in all slice preparations tested for 20, 30, 50, and 100 ms (Fig. 2). This indicates that neither infection with Ad-CMV-BDNF, Ad-CMV-NGF, or Ad-CMV-LacZ alter these basic properties of synaptic transmission in the slice, nor do any cytotoxic effects of the virus interfere with this process.

The main goal, of course, was to test whether LTP, which is severely compromised in BDNF-mutant mice (19, 33) can be restored by an adenovirus-mediated rescue with the BDNF gene. Indeed, when cells of the hippocampal CA1 region in slices from BDNF-mutant animals were infected with a Ad-CMV-BDNF the reduction of LTP in mutants could be largely counteracted. The amount of successfully induced LTP was increased 2.6-fold for [+/−] and 2.4-fold for [−/−] mice in

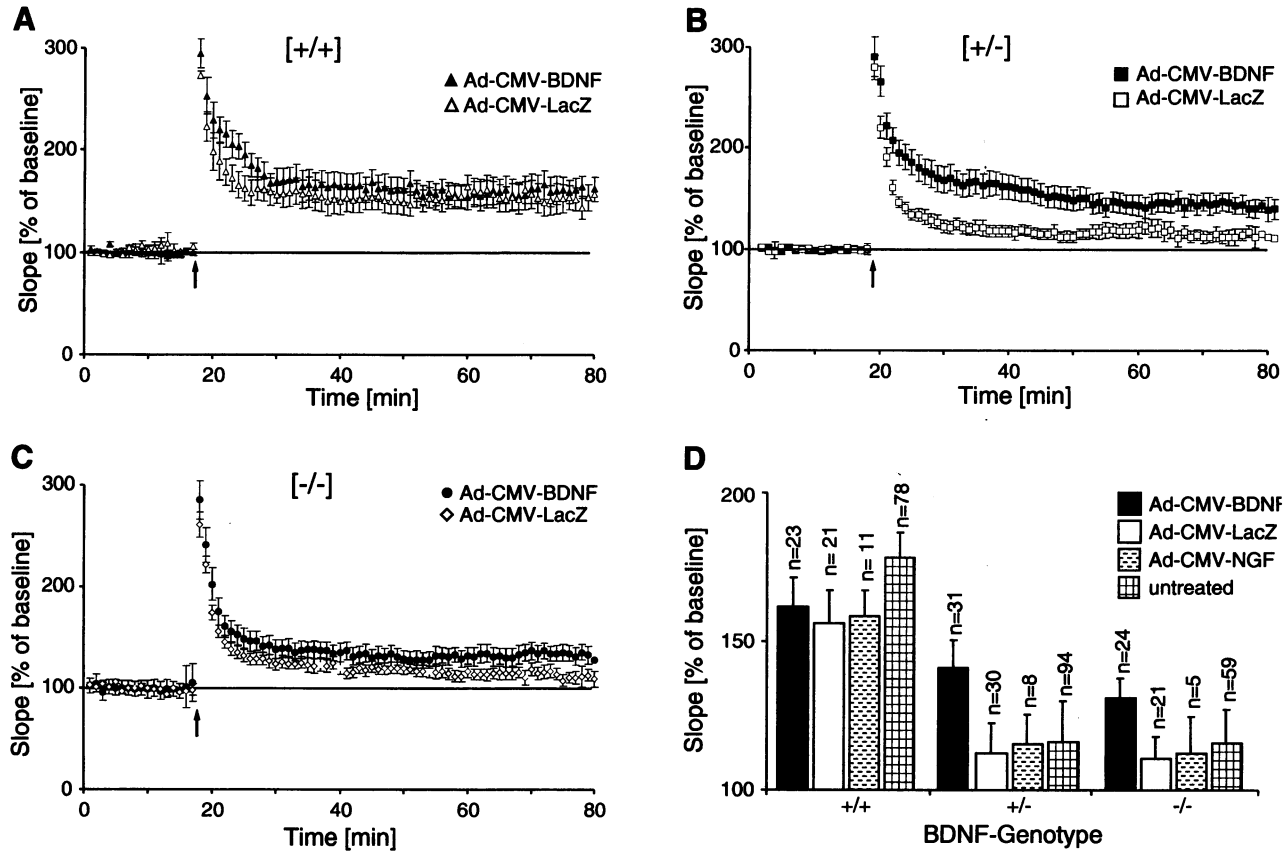

FIG. 3. Rescue of LTP by adenovirus-mediated gene transfer. (*A*) Ensemble average for all experiments in which Ad-CMV-LacZ (n = 21) or Ad-CMV-BDNF (n = 23) was injected into slices prepared from [+/+] mice. (*B*) Ensemble average for all experiments in which Ad-CMV-LacZ (n = 30) or Ad-CMV-BDNF (n = 31) was injected into slices from [+/−] mice. (*C*) Ensemble average for all experiments in which Ad-CMV-LacZ (n = 21) or Ad-CMV-BDNF (n = 24) was injected into [−/−] slices. Symbols represent mean over six responses. Arrows, time of tetanic stimulation (3 × 30 pulses, 100 Hz, 5 s ISI). (*D*) Summary graph for all genotypes with all different treatments (untreated control slices, NGF-, LacZ-, or BDNF-expressing adenoviral vector-injected slices). Slope values taken 60 min after tetanic stimulation. Enhancement is always shown with respect to baseline values. Error bars shown are SEM; n, number of slices.

comparison to Ad-CMV-LacZ injected slices (Table 1, compare also Fig. 3 *A–D*). In contrast, [+/+] mice showed almost no difference whether Ad-CMV-BDNF or Ad-CMV-LacZ was injected (Fig. 3 *A* and *D*). In the control case, when the infection was performed with Ad-CMV-NGF it did not result in any rescue of LTP in BDNF-mutant slices (Fig. 3*D*). For BDNF-deficient mice it becomes particularly evident in Fig. 3*D* that only Ad-CMV-BDNF injected slices show a significant increase in the EPSP slope 60 min after tetanic potentiation. Ad-CMV-NGF, Ad-CMV-LacZ, and untreated slices show no effect.

The average level of potentiation obtainable in [+/−] mice injected with Ad-CMV-BDNF is significantly higher than in [−/−] mice injected with the same virus construct ($P < 0.05$, two-tailed t test, compare Fig. 3 *B* with *C* and see Figs. 3*D* and 4*B*). This is most likely explained by a "threshold effect." [+/−] mice produce ≈50% of the normal amount of BDNF mRNA in comparison to wild-type mice (19, 34, 35). It is therefore reasonable to assume that in rescue experiments in [+/−] mice a critical threshold level of BDNF necessary for LTP (19) is reached more readily than in [−/−] mice.

The amount of potentiation observed even in [+/−] animals infected with Ad-CMV-BDNF never reached wild-type levels (compare solid squares with open triangles) (Fig. 4*A*). It is, of course, conceivable that a full rescue can never be achieved in animals that suffered from a lack of BDNF throughout development. There are however also a number of other factors that might prevent a full rescue. First, with recording field potentials it is to be expected that one also records from uninfected cells and therefore finds a somewhat lower potentiation in the rescue experiment. Moreover, it is likely that the conditions necessary for a good infection rate, most notably the raised temperature of 35°C for several hours and the mechanical manipulation of slices, cause a slight deterioration of the physiological state of the slice. This argument is supported by the results shown in Fig. 4, where it is clear that slices from [+/+] animals (shaded triangles) that underwent the infection protocol showed significantly reduced LTP as compared with untreated wild-type slices (see also Fig. 3*D*). There was no difference in the amount of potentiation or in the induction rate as to whether LTP was induced early or late after the virus injection, again supporting the view that the virus itself or its products are not cytotoxic within the incubation period.

Detailed analysis of the data shows that for [+/+] mice there are no statistically significant differences between slices injected with Ad-CMV-BDNF, Ad-CMV-NGF, or Ad-CMV-LacZ ($P > 0.35$, two-tailed t test), but for [+/−] mice the Ad-CMV-BDNF injected slices were significantly different from Ad-CMV-NGF ($P < 0.001$) and Ad-CMV-LacZ ($P < 0.001$) injected slices; [−/−] slices injected with Ad-CMV-BDNF were also significantly different from Ad-CMV-NGF ($P < 0.01$) and Ad-CMV-LacZ ($P < 0.01$) injected slices. For all experiments in all genotypes Ad-CMV-NGF injected slices were not statistically different from Ad-CMV-LacZ injected slices.

Despite the failure to reach full recovery of LTP the degree of rescue by the virus-mediated BDNF gene transfer is remarkable. This is most easily appreciated if the amount of potentiation observed in [+/−] and [−/−] mice is normalized

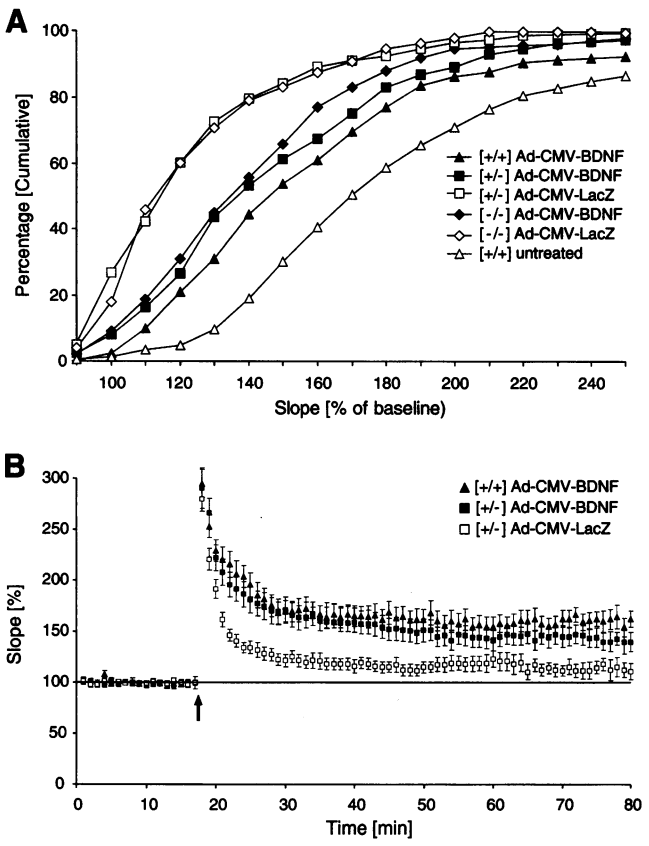

FIG. 4. (*A*) Cumulative percentage distribution of the post-tetanic field EPSP slopes, expressed as percentage of pre-tetanus values. All data points recorded 30–60 min after induction of LTP are included. The ordinate gives the fraction of the data points that exhibit a post-tetanic field EPSP slope less than or equal to the value shown on the abscissa. Data points of [+/−] and [−/−] BDNF-mutant mice, injected with Ad-CMV-BDNF or Ad-CMV-LacZ, and data points of untreated and Ad-CMV-BDNF injected [+/+] slices are shown. A shift to the left indicates less potentiation on average. The curves very quickly reach 100% on the *x*-axis for [+/−] and [−/−] mice injected with Ad-CMV-LacZ, indicating that very few slices show a substantial potentiation. In contrast, [+/−] and [−/−] slices infected with Ad-CMV-BDNF reach 100% later, i.e., many of them show a stronger potentiation. (*B*) Group data for field EPSP recordings before and after tetanic stimulation. This combination of group data shows that potentiation in Ad-CMV-BDNF injected slices from [+/−] mice almost reaches levels of wild-type slices injected with Ad-CMV-BDNF. In addition, the highly significant difference between Ad-CMV-LacZ and Ad-CMV-BDNF injected [+/−] slices becomes evident. Arrow, time point of tetanic stimulation; error bars shown are SEM.

to that observed in [+/+] mice that underwent the same procedures: the degree of potentiation then reaches 87 ± 11% in [+/−] mice (see Fig. 4*B*) and 81 ± 9% in [−/−] mice. In comparison, Ad-CMV-LacZ injected slices from [+/−] and [−/−] mice reach only 20% of the wild-type level.

To determine whether establishing the cellular sources of BDNF in presynaptic cells also results in a rescue effect we also infected cells in the CA3 region with the vectors. Under these circumstances we did not observe any rescue effect, i.e., the level of potentiation was statistically not different from LacZ adenoviral vector injection ($P > 0.05$, two-tailed t test).

DISCUSSION

Our data show that the compromised LTP in the CAT region of BDNF-knockout animals to undergo LTP can be rescued using adenoviral vectors to re-express the BDNF gene. This is further support for the notion that BDNF plays an important role in hippocampal LTP. These results differ in a number of important respects from recently published data, which also reported a rescue of LTP in BDNF-deficient animals (33). First, Patterson *et al.* (33) report that in their BDNF-knockout animals basal synaptic transmission and in particular PPF is severely affected. It is therefore not too surprising that LTP is also compromised in these animals. We tested basal synaptic transmission as well as PPF and found that—possibly due to the difference in genetic background—these parameters were not affected in our animals (see Fig. 2). These differences between the two studies suggest that the mechanism by which LTP is affected might be different in the different strains of BDNF-knockout mice. It is also important to note that Patterson and coworkers show in their rescue experiments that applying BDNF not only rescues LTP but also partly restores basal synaptic transmission (albeit with a different time course than LTP) arguing that the recovery in LTP might at least in part be due to improved synaptic transmission.

The second important difference between our paper and Patterson *et al.* (33) is that they incubated slices in BDNF, whereas we re-expressed the BDNF gene in cells that normally synthesize and release BDNF under physiological conditions. Our procedure therefore re-establishes a local cellular source for BDNF from which it can then be released in a constitutive and/or activity dependent manner (36). This seems particularly important in view of the fact that BDNF might act as a retrograde messenger. For this action, it is not unlikely that exogenously applied BDNF would act quite differently from cellularly released BDNF, just as it has been shown for the neurotransmitter glutamate, for which extracellular application can at best partially mimic synaptic release (e.g., ref. 37).

Our data show that virus-mediated gene transfer has to occur in CA1 cells and that re-expression of the gene in CA3 cells (which are presynaptic to CA1) is not sufficient to rescue LTP. These experiments have to be interpreted with caution, because it is difficult to ascertain that a sufficiently large fraction of CA3 neurons re-expressed BDNF. Nevertheless, they are compatible with the view that BDNF might act as a retrograde messenger, released by postsynaptic CA1 pyramidal neurons and acting on presynaptic terminals of CA3 cells as well as commissural fibers. This interpretation of a neurotrophin as a retrograde messenger is all the more attractive because it has been shown that neurotrophins can be released from dendrites in an activity dependent manner (38–41) and that they can enhance transmitter release (10, 11, 13). It is however also conceivable that neurotrophins act in an autocrine way on the cells from which they have been released, on neighboring CA1 cells, or on interneurons expressing the appropriate receptors (14, 42). Recent data showing that BDNF influences tetanus-induced LTP and that potentiation in adult hippocampal slices is decreased with function-blocking trkB-IgG receptor constructs (15) are in line with the results reported here, but also cannot distinguish between BDNF acting as a retrograde messenger or in an autocrine fashion.

Taken together our rescue experiments provide strong evidence that BDNF is causally involved in hippocampal LTP. The present results taken together with the data from other groups are compatible with the notion that BDNF could serve as a highly specific retrograde messenger for the induction of LTP. Moreover, our data contain the first demonstration that LTP, which has been shown to be impaired in a number of knockout animals (43), can be almost completely reinstated by re-expressing the ablated gene.

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